

Differential regulation of transcription and induction of programmed cell death by human p53-family members p63 and p73

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Abstract The p53 tumor suppressor acts as a transcription factor and has a central function in controlling apoptosis. With p63 and p73 two genes coding for proteins homologous to p53 have been identified. We describe the properties of seven human p63 and p73 proteins as transcriptional activators of *p21WAF1/CIP1* expression and apoptotic inducers in direct comparison to p53 in the same assay systems employing DLD-1-tet-off colon cells. Programmed cell death is detected in cells expressing high levels of p53 and p73 α . Cells overexpressing TAp63 α , TAp63 γ , TA*p63 α , TA*p63 γ , Δ Np63 α , and Δ Np63 γ display low or no detectable apoptosis. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

A principal factor in regulation of growth arrest as well as programmed cell death is the tumor suppressor protein p53. It acts by engaging in complexes with other proteins or functions as transcription factor [1–3]. It serves as an integrator of numerous signals affecting cell growth and apoptosis [1,4]. Many apoptotic signals are funnelled through p53 to the apoptotic machinery. Resistance to undergo programmed cell death in human cancers is often associated with loss of p53 function through mutation of its gene [4,5]. One example of the many genes on which p53 acts as a transcription factor is *bax* [6]. Induction of *bax* expression produces a protein which accelerates programmed cell death by counteracting the function of Bcl-2 [7]. Another prominent target for p53-dependent transcriptional activation is the cyclin-dependent kinase inhibitor *p21WAF1/CIP1* [2]. p53 is thought to exert its function in G₁ checkpoint control through p21 [8,9] and control over G₂/M transition through regulators like 14-3-3 σ [10], cdc25C [11], GADD45 [12], cyclin B [13] and cdc2 [14]. p53 also has a function in DNA repair which in part stems from its exonuclease activity [15,16].

With p63 and p73 two genes coding for proteins homologous to p53 have been identified [17–20]. Many studies inves-

tigated a possible function of the p53-related proteins as tumor suppressors. Nevertheless, there is still no final evidence that p73 is inactivated in tumors, and p63 displays even properties of an oncogene [21]. Both p63- and p73-deficient mice do not develop tumors [22–24]. However, p73 knockout mice display deficiencies in neurogenesis, sensory pathways and inflammation control [22] while p63-negative animals exhibit defects in limb and epidermal development [23,24]. Consistent with observations in the knockout mice mutations in p63 cause the human EEC syndrome which also results in limb malformation and other developmental deficiencies [25].

p63 and p73 proteins, in contrast to p53, are expressed in numerous splice variants making an analysis of their properties rather complex [21,26,27]. One evident question has been if any of the different p63 and p73 proteins can act as transcription factors and are able to induce programmed cell death like p53. A number of reports have addressed this issue [21]. However, a direct comparison of transcriptional and apoptotic characteristics of the different human p53 homologs under identical conditions was still lacking. We describe here, in the same assay systems, the properties of seven p63 and p73 proteins as transcriptional activators and apoptotic inducers in direct comparison to p53.

2. Materials and methods

2.1. Plasmids

Human p63 cDNA clones isolated from skeletal muscle and keratinocyte cDNA libraries provided sequence information for the major splice variants of p63 [28]. The protein coding for regions of TA*p63 α (GenBank acc. No. Y16961) [28], TA*p63 γ , Δ Np63 α (GenBank acc. No. AF 075431) [20] and Δ Np63 γ (GenBank acc. No. AF 075429) [20] were PCR-amplified from human tongue cDNA with specific primers including *Bam*HI and *Xba*I restriction sites. After subcloning of the PCR products into the appropriate sites of the eukaryotic expression vector pcDNA3.1/His A (Invitrogen) plasmids with correct reading frames were selected by sequencing. The cDNAs for p51A (TAp63 γ) and p51B (TAp63 α) were generously provided by Shuntaro Ikawa [19] and ligated as *Bam*HI/*Xho*I fragments into the pcDNA3.1/His C vector (Invitrogen).

The R175H mutation in p53 is often found in tumors and is unable to activate transcription [29]. For each p63 protein tested here, we selected and created expression clones that were mutated coding for His at the equivalent Arg position as in the p53 R175H mutant (K.R., unpublished). These DNA-binding mutants were created through PCR-mediated site-directed mutagenesis with the primers: p53R304H-For 5'-GAC GCT GCT TTG AGG CCC ACA TCT GTG CTT GCC CAG GAA G-3' and p51R304H-Rev 5'-TCC TGG GCA AGC ACA GAT GTG GGC CTC AAA GCA GCG TCG G-3'. This yielded mutant proteins with an Arg to His change

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in the DNA-binding domain which resembles R175H and p53 (TAp63 α and γ , R304H; Δ Np63 α and γ , R249H; TA*p63 α and γ , R343H). The human *p21* promoter construct, WWP-luc, and the human p53 expression plasmids, pCMV-p53wt and pCMV-p53mut, were generously provided by Bert Vogelstein [30,31]. The *p73* α wild-type and R273H mutant expression constructs have been described previously [11]. The control plasmid pRL-null (Promega) contains a cDNA coding for *Renilla* luciferase. All of the construct DNAs were purified through anion exchange columns (Qiagen) and confirmed by restriction analysis and sequencing.

2.2. Cell culture and stable transfection

SaOS-2 cells were obtained from DSMZ (Braunschweig, Germany) and cultured in a humidified atmosphere with 7.5% CO₂ at 37°C. Inducible cell lines D.P53 A2, 175 A4, sigma A4 and HA-wt-p73- α 292 were kindly provided by Bert Vogelstein. All four cell lines are derivatives of the colorectal carcinoma cell line DLD-1 which has endogenous mutant p53 alleles [32]. They were cultured as described previously [13]. For p63-inducible cell lines the cDNAs of p63 were cloned in pBI-MCS-EGFP [32]. DLDtet14 cells were cotransfected with these constructs and pTK-Hyg (Clontech) and selected for stable transfected cells by growing in 10% FCS in McCoy's 5A modified medium (Biochrom) containing 400 μ g/ml geneticin (Gibco BRL) and 250 μ g/ml hygromycin (Roche). Expression of *p53*, *p63* or *p73* is regulated by a modified tetracycline (tet)-regulated gene expression system (tet-off system) [13,32,33].

2.3. Transfection and luciferase assays

Transfections of SaOS-2 cells were done as described [13]. 250 ng of luciferase reporter constructs were cotransfected with 25 ng of constructs coding for wild-type or mutant p53, p63 or p73 and 25 ng or pRL-null vector (Promega) as a transfection control. Firefly and *Renilla* luciferase activities were assayed with the Dual Luciferase Assay System (Promega) [34]. The firefly luciferase activity was normalized to *Renilla* luciferase activity to compensate for variability in transfection efficiencies [11].

2.4. Western blotting, RNA extraction and LightCycler RT-PCR analysis

Western blot analyses using 25 μ g of protein per lane were performed as described [13] and analyzed with the ECL Western blotting analysis system (Amersham). The antibodies employed were the anti-p73 mouse monoclonal antibody Ab-1 (Oncogene) and the anti-p63 mouse monoclonal antibody 4A4 (Santa Cruz). Extraction of total RNA and RT-PCR mRNA quantification with the LightCycler system including calculations have been described [11,13].

2.5. DNA ladder and fluorescence-activated cell-sorting (FACS) analysis

Adherent and floating cells were collected after indicated time points following removal of doxycycline from the medium. DNA was purified from these cells using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's suggestions. 2 μ g of total DNA were separated for each sample after staining with

SYBR Green (Biozym) on 1.6% agarose gels. For FACS analyses cells, both floating and adherent, were harvested at indicated time points after induced expression of p53, p63 or p73, respectively, washed twice in PBS/EDTA (1 mM) and fixed with 80% ethanol in PBS/EDTA for at least 48 h at 4°C. Cells were centrifuged and resuspended in 1 ml PBS/EDTA containing 50 μ g/ml RNase A (Sigma), stained with propidium iodide (Sigma) at a final concentration of 60 μ g/ml. A total of 25000 cells were analyzed by flow cytometry as described [35].

2.6. Caspase-3 assay

p53, p63 or p73 expression, respectively was induced and floating as well as adherent cells were collected at indicated time points after induction. Caspase-3 activity was assayed with the caspase-3 colorimetric assay kit (Sigma) according to manufacturer's instructions. The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the pNA moiety. The 96-well assay plate was incubated for 90 min at 37°C before measurements were taken. The results were calculated using a pNA calibration curve.

3. Results

The p53-family members included in this study were p53, TAp63 α , TAp63 γ , TA*p63 α , TA*p63 γ , Δ Np63 α , Δ Np63 γ and p73 α (Fig. 1). Experiments studying both transcriptional properties and induction of apoptosis were controlled in parallel experiments by expression of mutant variants of these proteins which were deficient in DNA binding.

3.1. Induction of transcription by p53, p63 and p73

Activation of *p21WAF1/CIP1* transcription resulting from expressing different p53-family members was employed as an assay for transcriptional activity and was tested in two ways. Expression of mRNA from the chromosomal *p21* gene was monitored by RT-PCR in the colorectal adenocarcinoma cell line DLD-1 after tet-off induction of p53-family members. The second way was to employ in SaOS-2 osteosarcoma cells a *p21*-promoter reporter construct by cotransfections with p53-family member-expressing plasmids.

Expression of *p21* by p53 results in the strongest induction in both the mRNA levels and reporter expression (Table 1). Among the p53 splice variants tested TA*p63 α , Δ Np63 α and Δ Np63 γ did not display any significant induction of *p21* transcription. TA*p63 γ induced the *p21* reporter but did not lead to a significant expression of *p21* mRNA when expressed in DLD-1 cells. A small induction of *p21* chromosomal gene

Table 1

Induction of *p21WAF1/CIP1* mRNA expression and *p21* promoter-driven luciferase reporter activity after transactivation through p53-family members

	p21 endogenous mRNA induction factor	p21 promoter reporter induction factor
p53	25.9	3.9
TAp63 α	2.9	1.3
TAp63 γ	14.4	3.2
TA*p63 α	1.2	1.2
TA*p63 γ	1.2	3.7
Δ Np63 α	1.3	1.5
Δ Np63 γ	1.3	1.2
p73 α	3.8	3.5

mRNA levels were measured from DLD-1 colorectal adenocarcinoma cells stably transfected with tet-off vectors expressing members of the p53 family. *p21* mRNA levels derived from chromosomal genes were determined by LightCycler RT-PCR and standardized with *GAPDH* mRNA concentrations. Ratios of normalized *p21* mRNA levels after induction to levels before induction of p53-family member expression are given as induction factor. *p21* promoter-dependent luciferase activity was measured in SaOS-2 cells after cotransfection of the *p21*-promoter reporter construct with expression vectors carrying wild-type or mutant cDNAs of p53-family members. Ratios of luciferase activities from experiments with wild-type versus DNA-binding mutant p53-family members are given as induction factors. Each experiment was done in triplicate with averages given in the table.

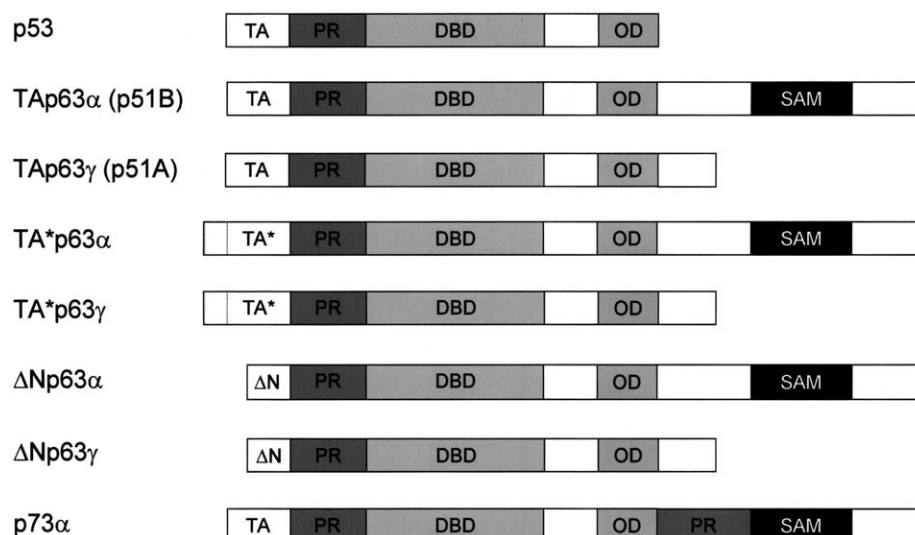


Fig. 1. Nomenclature and domain structure for p53, p73α and p63 splice variants. TA, transactivation domain; TA*, transactivation domain including a 39 amino acid extension; ΔN, truncated amino terminal domain; PR, proline-rich domain; DBD, DNA-binding domain; OD, oligomerization domain; SAM, sterile α-motif domain.

expression was observed when TAp63α was overexpressed. However, the *p21*-reporter assay yielded no significant increase after TAp63α expression. In contrast, TAp63γ expression resulted in clear transactivation after employing both assay systems. Also p73α led to a significant *p21* expression in both assay systems (Table 1).

Expression of p53-family members on the mRNA level (data not shown) and on the protein level, as controls, were checked by Western blotting (Fig. 2). All p53-family members appear with increased protein expression after tet-off induction. Protein expression of p53 and p73α and DLD-1-tet-off cells had been described previously [11,13,32]. All p63 and p73 splice variants are expressed on a base level from their chromosomal genes as detected by RT-PCR already in uninduced DLD-1-tet-off cells (data not shown). This is consistent with the observation that also some proteins are observed on a basal level even in uninduced DLD-1-tet-off cells (Fig. 2).

3.2. Programmed cell death in cells expressing high levels of p53 and p73α but low or no detectable apoptosis in cells overexpressing TAp63α, TAp63γ, TA*p63α, TA*p63γ, ΔNp63α and ΔNp63γ

The ability of the p53-family members to induce programmed cell death in a DLD-1-tet-off cell system was tested by DNA laddering, caspase-3 assays and flow cytometry. A classical test to evaluate induction of programmed cell death is to look at fragmentation laddering of chromosomal DNA in agarose gels as a late marker for programmed cell death. In this assay, which may be the least sensitive of the three assays employed, only induction of p53 in the colorectal carcinoma cells led to laddering usually associated with apoptosis (Fig. 3). Neither p73α nor any p63 homolog resulted in DNA fragmentation even after induced expression for 96 h (Fig. 3 and data not shown).

The strongest induction of caspase activity, as a marker for

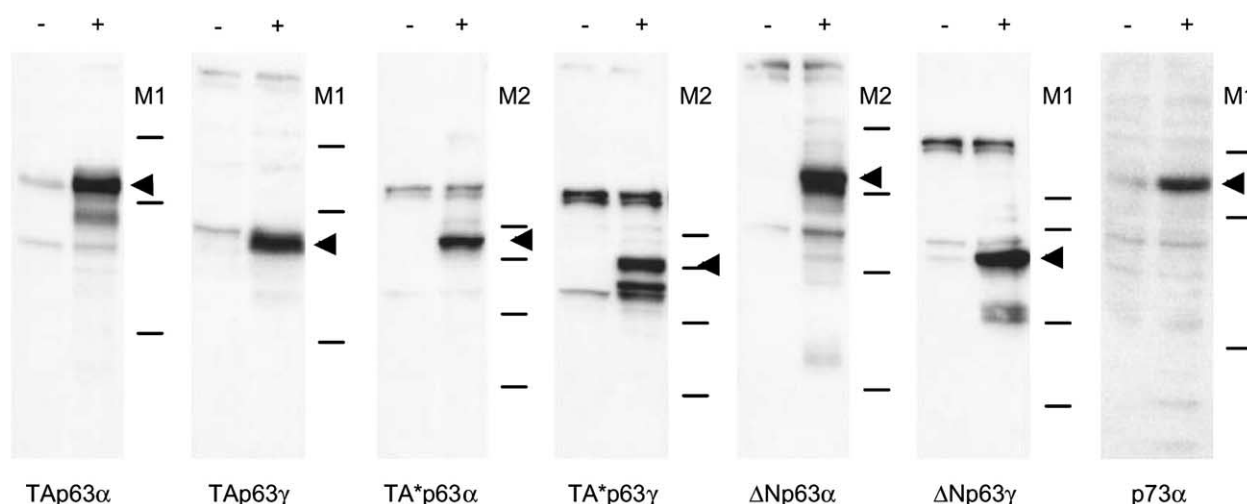


Fig. 2. Western blot analysis controlling expression of different p63 and p73 proteins. Lysates from uninduced (–) and induced (+) tet-off DLD-1 cells with the indicated transgenes were analyzed with antibodies directed against their protein products. Two kinds of molecular weight markers were employed: M1: (27, 39, 66 and 97 kDa; M2: 35, 50, 75 and 105 kDa).

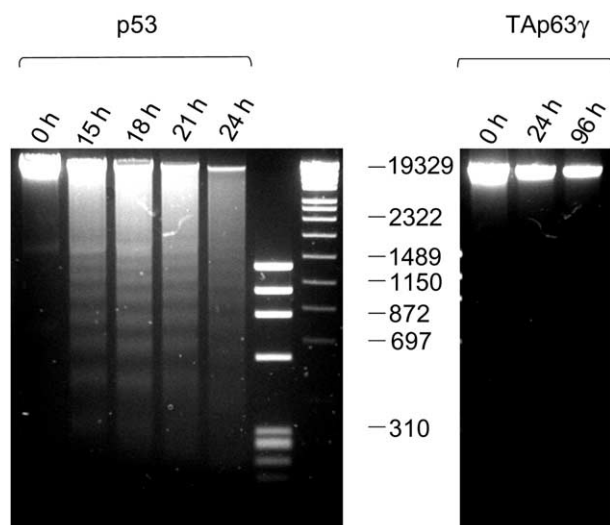


Fig. 3. Fragmentation laddering of chromosomal DNA in agarose gels after induced expression of p53 and TAp63 γ in DLD-1-tet-off cells. Experiments were done in parallel. Time points are indicated for analyses performed before and after release of the tet-off system. 2 μ g of DNA were extracted from suspensions for each experiment to be loaded on the agarose gel. Molecular weight standards indicating number of base pairs are given between the two gels.

an early stage of apoptosis, is seen by p53. We observe a steep increase in caspase activity already before 15 h of induced expression of p53. It reaches peak levels after 24 h at which time a large number of cells were already lost by apoptosis (Fig. 4). Measurements at times later than 24 h were not useful since hardly any viable cells were left after 24 h (data not shown). Induction of p73 α expression also resulted in a strong caspase activity. In this case caspase activity was measured up to 48 h before a large portion of the cells had died. None of the p63-family members yielded an increase in caspase activity when overexpressed, even after 96 h (Fig. 4).

The tet-off cells were also used to assess the ability of p53-family transgenes to induce programmed cell death as judged by the percentage of sub-G₁ cells in flow cytometry. FACS

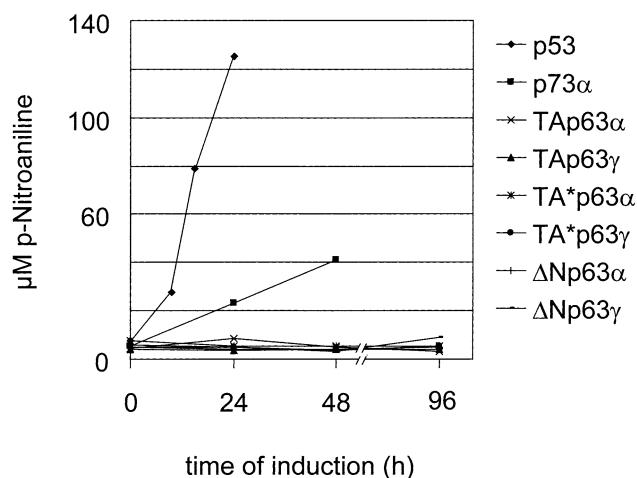


Fig. 4. Caspase activity in lysates from DLD-1-tet-off cells expressing high levels of p53-family members. pNA was measured as product formed from caspase-3 activity. Lysates were taken at indicated time points after induction of the tet-off system.

Table 2

Sub-G₁ cell populations from FACS analyses of DLD-1-tet-off cells before and after induction of transgenes

	0 h	24 h	48 h	96 h
p53wt	3.5	23.1	n.a.	n.a.
p53mut	3.1	3.4	3.0	–
TAp63 α	2.5	3.0	6.0	10.3
TAp63 γ	2.5	3.3	6.2	11.2
TA*p63 α	0.8	0.5	1.2	1.1
TA*p63 γ	1.7	2.1	2.3	1.9
Δ Np63 α	0.7	0.8	0.9	0.8
Δ Np63 γ	1.8	1.8	2.4	2.8
p73 α	2.6	8.1	40.3	n.a.

Percentages of cell populations are given gated for cells with a DNA content less than that found in G₁ cells. N.a., not applicable; total number of surviving cells too low. Full diagrams for some of the experiments are shown in Fig. 5. For DLD-1-tet-off-p53, wild-type and a DNA-binding mutant, p53R175H, are shown. The other constructs all code for the wild-type proteins.

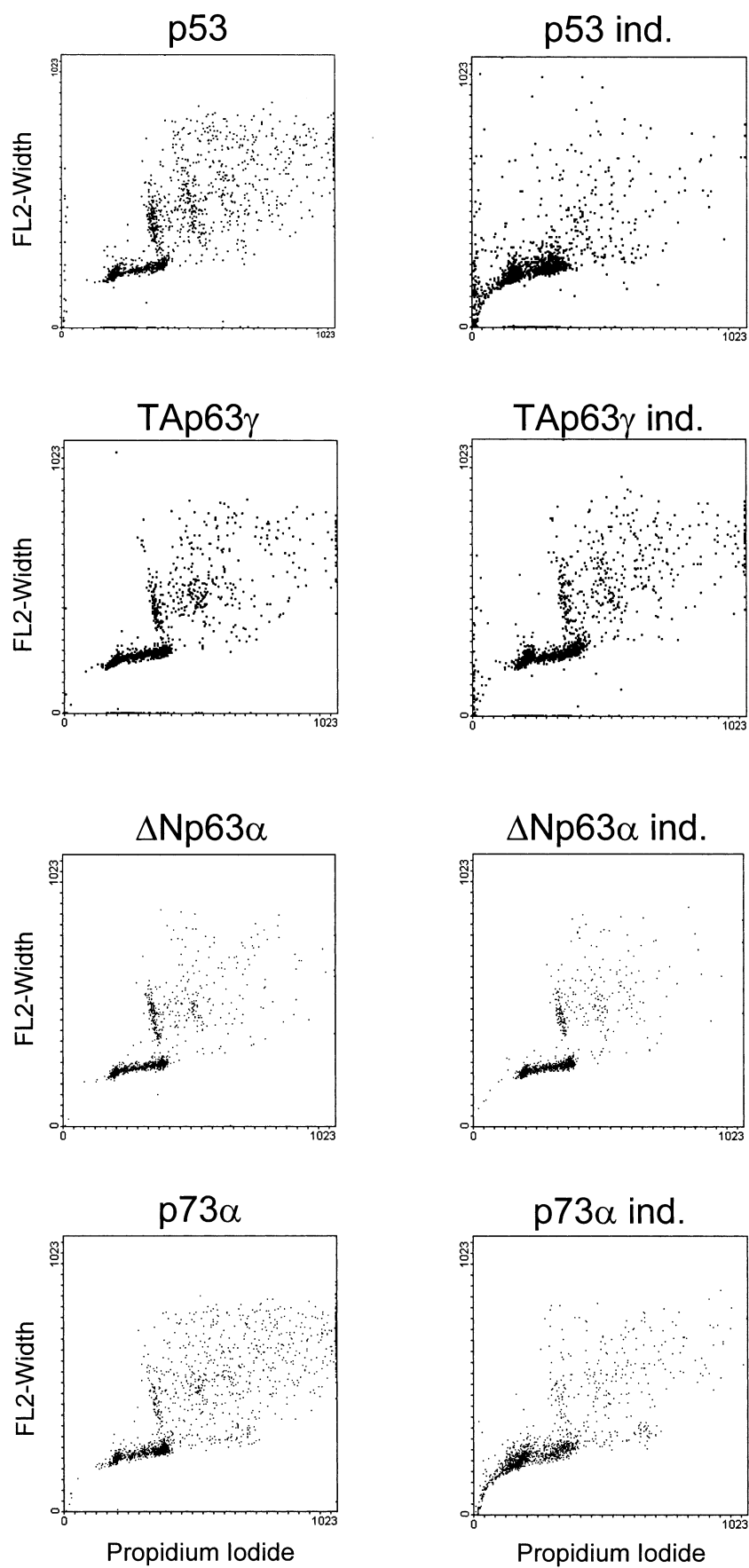
analyses looking at DNA staining per cell were performed with cells before and after tet-off-induction. Again, wild-type p53 expression gave strong signals of apoptosis already after 24 h (Fig. 5 and Table 2). In contrast, the DNA-binding domain mutant p53R175H did not display any ability to induce apoptosis. Similarly, expression of TA*p63 α , TA*p63 γ , Δ Np63 α and Δ Np63 γ did not lead to apoptosis even 96 h after induction. However, the other two p63 forms, TAp63 α and TAp63 γ , yield a weak apoptosis measured at 48 to 96 h (Table 2). p73 α overexpression leads to a clear induction of programmed cell death as judged by appearance of a sub-G₁ cell population (Fig. 5 and Table 2). This is even more significant considering that the actual level of apoptosis is underestimated by this assay since fragmentation of DNA in G₂- and M-phase cells first leads to an increase of cell populations apparently in G₁- and S-phases.

4. Discussion

Following the discovery of the p63 and p73 families, understanding of the p53 network regulating checkpoint control, transcription of target genes and programmed cell death has become more challenging. It has been documented that the new classes of proteins cannot simply replace p53 as a tumor suppressor [21,26,36]. In this report we describe the transcriptional properties and the ability to induce apoptosis in the same assay systems for seven p63 and p73 proteins in direct comparison to p53.

The well established induction of p21 expression by p53 served as an indicator to judge the transcriptional activity of various p53-family members in DLD-1 and SaOS-2 cells, which are both functionally negative for p53. Two kinds of assays were employed, one including stably transfected DLD-1 colon cells measuring mRNA levels from the chromo-

Fig. 5. FACS analyses of DLD-1-tet-off cells before and after induction of transgenes. Four examples of the DLD-tet-off cells are shown. A complete list with gated percentages of sub-G₁ populations from the experiments are given in Table 2. Cells were harvested before removal of the antibiotic as a control (left column) or after elimination of doxycycline from the medium (right column; 24 h for p53 and 48 h for the other proteins, respectively). Cells were stained with propidium iodide and subjected to flow cytometry. DNA staining versus FL2 width is depicted.



somal *p21* gene and the other assay monitoring a *p21*-promoter reporter in transiently transfected SaOS-2 osteosarcoma cells (Table 1). The strongest *p21* expression resulted from induction of wild-type p53. Also p73 α and TAp63 γ yielded a clear *p21* induction in both assays. TA*p63 α , Δ Np63 α and Δ Np63 γ were negative for an induction of *p21* transcription. The results for p53, TAp63 γ and p73 are consistent with previous observations although we find a more significant activation of transcription by p53 compared to p73 in stably transfected colon cells [17,19,37]. TA*p63 γ shows an induction of the *p21* reporter but does not increase *p21* mRNA levels (Table 1). A low stimulation of a p53-responsive reporter by TA*p63 γ has also been observed by Yang et al. [20] and Bamberger and Schmale [27]. Transcriptional tests using expression from the chromosomal gene appear to be better systems since they are closer to the situation in a normal cell. Therefore, we suggest to consider TA*p63 γ as not being able to induce *p21* transcription. Transcriptional regulation by TAp63 α has been controversial with Yang et al. [20] interpreting the influence on an artificial p53-responsive promoter as not significant and Dohn et al. [38] finding an induction of endogenous *p21* gene and a *p21*-promoter construct. Our results are closer to those by Dohn et al. (Table 1). Also the observation by Yang et al. could be interpreted as a weak induction of transcription by TAp63 α since they found an increase higher than the control but not as strong as with TAp63 γ and p53 [20]. The remaining difference may be due to their use of mouse cDNAs in a human cell system whereas we employed both cells and cDNAs of human origin.

Induction of apoptosis was monitored by DNA laddering, caspase-3 assays, and measuring sub-G₁ populations with flow cytometry after overexpression of the wild-type p53-family members in colon cells (Table 2, Figs. 3–5). We used DNA-binding mutants for each p53-family member in the DLD-1 system as negative controls in the apoptosis assays. None of the mutant proteins yielded a significant number of apoptotic cells (data not shown). The three assays revealed that p53 gives the strongest induction of programmed cell death. Also p73 α could clearly induce apoptosis. TA*p63 α , TA*p63 γ , Δ Np63 α and Δ Np63 γ were negative for induction of programmed cell death. TAp63 α and TAp63 γ showed a moderate increase in the sub-G₁ cell population indicating apoptosis. Results for p53, TAp63 γ , TA*p63 α , TA*p63 γ , Δ Np63 γ and p73 α were consistent with previous observations in different cell and assay systems [20,37,38]. However, the induction of programmed cell death at a similar level by TAp63 α and Δ Np63 α seen by Dohn et al. [38] is in contrast to results by Yang et al. [20] and observations described here. We find a moderate increase of sub-G₁ cells after 2–4 days of stimulated TAp63 α and TAp63 γ expression (Table 2). Δ Np63 α does not induce apoptosis as also seen by Yang et al. [20]. The different results may be due to the different cell and assay systems employed in these studies. Also the published results provide information on only a few of the proteins studied here.

Generally, we provide for the first time directly comparable results for a set of eight human p53-family members on *p21* transcriptional regulation and on apoptosis induction obtained with identical assay conditions. In most cases in which we observe *p21* expression stimulated by the overexpressing of one p53-family member we also see a correlation with apoptosis induction. This fits the idea that the cell cycle inhibitor

p21WAF1/CIP1 is important for the G₁/S and G₂/M checkpoint arrests which are often seen as prelude to programmed cell death [8,39].

For future work a better understanding of the interactions between p53-family members is needed. Recently, a report using mice deficient in different combinations of intact p53, p63 and p73 genes showed that apoptosis induction by p53 after DNA damage is dependent on p63 and p73 [40]. Another direction for further experiments is to elucidate how different transcriptional target genes are regulated by proteins of the p53 family. It has been noticed that members of the p53 family display differences in transcriptional activity towards the same target genes [11,32,38].

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